Amendments to the Specification

Page 1, line 2, insert the following:

- - This application is a national stage application, filed under 35 U.S.C. 371, of PCT application number PCT/IB03/01791 filed on April 9, 2003, and US provisional priority application serial number 60/370,690, filed on April 9, 2002. - -

Page 3, replace the paragraph beginning at line 33 with the following:

-- The method according to the invention provides a means for the analysis of complex methylation patterns within biological samples by use of multiple pairs of methylation specific primers. After unmethylated cytosine bases in a nucleic acid sample are converted into uracil bases, which is ~~provided~~ accomplished by a converting agent that does not change methylated cytosines, selected segments of the converted nucleic acid sample are amplified in a polymerase reaction wherein at least two oligonucleotide primer pairs are employed, such that the amplificates formed are differentially detectable and quantifiable. One primer pair binds preferentially to treated nucleic acid that was initially methylated in the sequence the primer is hybridizing to. Another primer pair binds preferentially to treated nucleic acid that was initially unmethylated in the sequence the primer is hybridizing to. A third oligonucleotide primer pair may be used to amplify a sequence that acts as a reference sequence. The degree of methylation in at least one selected segment of the nucleic acid sample is determined based on comparative differences in amplificates formed from each of the oligonucleotide primer pairs. The invention also includes primers and a kit. - -

Page 4, between lines 14 and 15 insert:

- - Fig. 3 is a flow chart illustrating yet another preferred embodiment of the invention. - - .

Page 7, replace the paragraph beginning at line 1 with:

-- The extraction of DNA for further analysis can take place in a minute volume, usually in a layer of oil, which prevents contact with the environment and [keep] keeps losses of DNA low to provide a reproducible result even with small starting quantities. --

Page 8, replace the paragraph beginning at line 29 with:

-- According to one embodiment of the invention, each pair of amplification primers consists of a first (forward) primer and a second (reverse) primer, as [in] is standard in many amplification methods, such as polymerase chain reaction PCR). Each of the primer pairs is required to consist of at least one methylation specific primer oligonucleotide. A methylation specific primer refers to a primer oligonucleotide for use in the amplification of a methylation discriminating bisulfite treated nucleic acid (or similarly converted nucleic acid), wherein the primer contains at least one CpG or CpA dinucleotide within its sequence. As described in, for example, U.S. Pat. No. 5,796,146 to Herman et al., MSP primers consist of an oligonucleotide specific for annealing to a nucleotide sequence containing at least one bisulphite treated CpG dinucleotide. Therefore, according to this embodiment of the method, the primer pair that hybridizes preferentially to the target nucleic acid that was methylated prior to the bisulfite treatment comprises a CpG dinucleotide at the CpG position to be investigated, and the primer pair that hybridises preferentially to the target nucleic acid that was unmethylated prior to the bisulfite treatment comprises a TpG or CpA dinucleotide at the CpG position. Methylation specific primers generally contain relatively few cytosines, as cytosines are converted by the bisulphite reaction. However, when the primers are specific for methylated cytosine dinucleotides, cytosine positions are conserved within the primer oligonucleotides. Therefore, the sequence of the primers includes at least one CpG, TpG, or CpA dinucleotide. ~~MSP primers generally contain relatively few cytosines, as cytosines are converted by the bisulphite reaction. However, when the primers are specific for methylated cytosine dinucleotides, cytosine positions are conserved within the primer oligonucleotides.~~ --

Page 9, replace the paragraph beginning at line 30 with:

- - In a preferred embodiment, all primers are then extended, preferably by means of a polymerase reaction. ~~Wherein~~ When a polymerase is used, the resultant double-stranded nucleic acid is denatured, preferably by means of heat treatment. Successive cycles of primer annealing, extension, and denaturation are carried out according to the polymerase chain reaction, as is known in the art. - -

Page 11, replace the paragraph beginning at line 27 with:

- - A particularly preferred embodiment of this method is the use of fluorescence-based Real Time Quantitative PCR (Heid et al., Genome Res. 6:986-994, 1996; see also U.S. Pat. No. 6,331,393 B1, Laird et al.). There are two preferred embodiments of utilising this method. One embodiment, known as the ~~TaqMan™~~ TAQMAN® assay employs a dual-labelled fluorescent oligonucleotide probe. The ~~TaqMan™~~ TAQMAN® PCR reaction employs the use of a nonextendible interrogating oligonucleotide, called a ~~TaqMan™~~ TAQMAN® probe, which is designed to hybridise to a target sequence located between the forward and reverse amplification primers. The ~~TaqMan™~~ TAQMAN® probe further comprises a fluorescent "reporter moiety" and a "quencher moiety" covalently bound to linker moieties (e.g., phosphoramidites) attached to the nucleotides of the ~~TaqMan™~~ TAQMAN® oligonucleotide. Hybridized probes are displaced and broken down by the polymerase of the amplification reaction thereby leading to an increase in fluorescence. For analysis of methylation within nucleic acids subsequent to bisulfite treatment, it is further preferred that the probe be methylation specific, as described in Laird et al. (hereby incorporated by reference in its entirety), also known as the ~~MethyLight™~~ MethyLight assay. The second preferred embodiment of this technology is the use of dual-probe technology (~~Lightcycler™~~ LIGHTCYCLER®). Each probe carries a donor or recipient fluorescent ~~moieties~~ moiety, hybridization of two probes in proximity to each other is indicated by an increase [or] of fluorescent

amplification primers. This technique may also be adapted in a manner suitable for methylation analysis of CpG dinucleotides within the amplificates. - -

Page 15, replace the two paragraphs beginning at line 27 with:

- - DNA may be extracted using a suitable commercially available kit e.g. ~~Qiagen™~~ QIAGEN® extraction kit. The DNA sample is then treated using a bisulfite solution (hydrogen sulfite, disulfite) according to the agarose-bead method (Olek et al 1996). The treatment is such that all non methylated cytosines within the sample are converted to thymidine. Conversely, 5-methylated cytosines within the sample remain unmodified. The methylation status is determined with a methylation specific assay designed for the CpG island of interest. The CpG island assay covers CpG sites in both the primers and the ~~Taqman™~~ TAQMAN® style probe. Methods:

The assay specific to the methylated version of the CpG island is performed using the following primers and probes: [0064] Primer: TTTTCGTCGTTTAGGTTATCG (SEQ ID NO:1); Primer: TTTTTGTTGTTTTAGGTTATTGG (SEQ ID NO:2); and Probe: TTCGGACGTCGTTGTTCGGTCGATGT (SEQ ID NO:3). The corresponding assay specific to the unmethylated version of the CpG island is performed using the following primers and probes: Primer: TTTTTGTTGTTTTAGGTTATTGG (SEQ ID NO:4); Primer: CATATGCTGTGAATAAATTAC (SEQ ID NO:5); and Probe: TTTGGATGTTGTTGTTTGGTTGATGT (SEQ ID NO:6) The reaction is run with the following assay conditions: Reaction solution: (900 nM primers; 300 nM probe; 3.5 mM Magnesium Chloride; 1 unit of taq polymerase; 200 µM dNTPs; 7µl of DNA, in a final reaction volume of 20 µl); Cycling conditions: (95° C for 10 minutes; then 50 cycles of: 95 ° C for 15 seconds; 60 ° C for 1 minute). The reaction is observed in real time by use of commercially available instruments such as the ABI PRISM® 7700 sequence detector. - - .

Page 16, replace the paragraph beginning at line 20 with:

- - DNA may be extracted using a suitable commercially available kit, e.g. ~~Qiagen™~~ QIAGEN® extraction kit. The DNA sample is then treated using a bisulfite solution (hydrogen sulfite, disulfite) according to the agarose-bead method (Olek et al 1996). The treatment is such that all non methylated cytosines within the sample are converted to thymidine. Conversely, 5-methylated cytosines within the sample remain unmodified. The methylation status may be determined with a methylation specific assay designed for the CpG island of interest and a reference fragment . - - .

Page 17, replace the paragraph beginning at line 1 with:

- - The corresponding reference assay was performed using the following primers and probes: Primer: TCCATATTCCAAACCCTATACCAAA (SEQ ID NO:13); Primer: TGGGATTGAGGGTAAGAGGGAT (SEQ ID NO:14). The reaction is run with the following assay conditions: Reaction solution: (900 nM primers; 300 nM probe; 3.5 mM Magnesium Chloride; 1 unit of taq polymerase; 200 µM dNTPs; 7µl of DNA, in a final reaction volume of 20µl); Cycling conditions: (95 ° C for 10 minutes; then 50 cycles of: 95 ° C for 15 seconds; 60 ° C for 1 minute ). The reaction is observed in real time by use of commercially available instruments such as the ABI PRISM® 7700 sequence detector. - -.

Page 17, replace the paragraph beginning at line 25 with:

- - A first sample is obtained from a tumor sample, and is isolated using a suitable commercially available kit e.g. ~~Qiagen™~~ QIAGEN ® extraction kit. - - .

Page 18, replace the paragraph beginning at line 21 with:

- - Each reaction is run with the following assay conditions: Reaction solution: (900 nM primers; 300 nM probe; 3.5 mM Magnesium Chloride; 1 unit of taq polymerase; 200 μM dNTPs; 7μl of DNA, in a final reaction volume of 20μl); Cycling conditions: (95° C for 10 minutes; then 50 cycles of: 95 ° C for 15 seconds; 60 ° C for 1 minute ). The reaction is observed in real time by use of commercially available instruments such as the ABI PRISM® 7700 sequence detector. The amount of methylated nucleic acid in the tumor sample is quantified by plotting a first calibration curve wherein the amount of amplificate from each sample is plotted against cycle number. From this plot, the crossing line is determined, the crossing line being the point on each curve at which the PCR cycle amplification signal enters the log linear phase. Using these intersection points a calibration graph can be calculated which defines a relationship between the cycle number at which the amplification signal intersects the crossing line and the template concentration initially present in the sample. Thus, if the intersection point of an amplification signal (expressed as the cycle number) is known, the initial template concentration can be directly derived from the calibration graph. These calculations may be calculated using the Fit Points and Second Derivative Maximum Methods. The Second Derivative Maximum Method is preferred if samples with a high copy number (above 1000 copies/sample) are to be analyzed. If samples with a low copy number are to be analyzed, the Fit Points Method is preferred. - - .